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Genetic transformation of *Streptococcus thermophilus* by electroporation

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Summary — A rapid and convenient electroporation procedure was developed for the genetic transformation of intact cells of *Streptococcus thermophilus* with various species of plasmid DNA. Transformation frequency was influenced by the capacitance and voltage selected for electric pulsing, the pH and composition of the electroporation medium and the molecular mass of the transforming DNA. Electroporation is a simple and effective technique to introduce plasmid DNA into *S. thermophilus* and useful in the development of recombinant DNA technology for this important industrial microorganism.

electroporation / *Streptococcus* / genetic transformation

Introduction

Streptococcus thermophilus is an industrially important species and is widely used in dairy fermentations, most frequently in combination with lactobacilli and mesophilic streptococci. Although cloning vectors suitable for genetic studies in streptococci are available [1–4], direct insertion of DNA is possible only in competent cells of clinically important streptococci with a functional genetic transformation apparatus [5, 6], that is apparently absent in the food-fermenting streptococci. DNA has been successfully introduced into protoplasts of lactic streptococci with the aid of polyethylene glycol [7, 8], but regeneration of viable cells from protoplasts is often a slow and inefficient process. These problems have, in part, contributed to the slow progress in the development of recombinant DNA techniques suitable for *S. thermophilus*.

It is known that exposure to a pulsed electric field results in the transient permeabilization of different types of cells allowing the uptake of DNA presumably through pores created in the cytoplasmic membrane [9]. Electroporation has been used to introduce DNA into plant protoplasts [10–12] and animal cells [13–15], proto-

plasts of *Saccharomyces cerevisiae* [16], *Bacillus cereus* [17] and *Streptomyces lividans* [18], as well as intact cells of *S. cerevisiae* [19], *Streptococcus lactis* [20], *Escherichia coli* (W. Dower and K. Shigekawa, personal communication), and most recently, into *Lactobacillus casei* [21].

In this paper, we describe the electroporation-dependent transformation of *S. thermophilus* and the effect of conditions influencing the uptake of plasmid DNA up to 30 kilobases (kb) in molecular mass by intact cells of this organism. (This material was presented in part at the 4th European Congress on Biotechnology, 14–19 June 1987, Amsterdam, The Netherlands.)

Materials and methods

Microbial cultures and growth conditions

Strains of *Streptococcus thermophilus* studied included carriers of plasmid DNAs [22] and plasmid-free cultures (Table I). Cultures were routinely maintained in a lactose broth medium [23] at 37°C and transferred once every 7 days. Between transfers, cultures were stored at 4°C.

Table I. *Streptococcus thermophilus* strains used in electroporation.

Strain	Plasmid ^a	Size, kb	Phenotype	Source ^b
ST101				Hansen
108				Hansen
120	pER8	2.20	unknown	NRRL
ST113	pER13	4.23	unknown	Microlife
ST116				Miles
126	pER16	4.46	unknown	Uneb
ST134	pER341	2.70	unknown	Nestle
	pER342	9.50	unknown	
ST136	pER36	3.70	unknown	Nestle
ST102				Hansen
104				Hansen
110				Microlife
128	none			Nestle
132				INRA
133				unknown

^a[22].

^bHansen: Chr. Hansen's Laboratory, Inc.; NRRL: Northern Regional Research Laboratory, USDA; Microlife: Microlife Technologies; Miles: Miles Laboratories, Inc.; Uneb: University of Nebraska; Nestle: Nestle Products, Ltd., Lausanne, Switzerland; INRA: National Agricultural Research Institute, Jouy-en-Josas, France.

Transforming DNA

The erythromycin resistance (Em^r) plasmid pVA736 (7.6 kb) constructed by Macrina *et al.* [4] from *EcoRI*/*HindIII* fragments of the cryptic plasmid pVA380-1 of *S. ferus* and the Em^r plasmid pVA1, a spontaneous deletion mutant of pAMβ1 of *S. faecalis*, was used as the transforming vector DNA in most electroporation experiments. From the host *S. sanguis* (Challis), pVA736 was isolated by a rapid procedure [24], followed by purification in a cesium chloride gradient as described by Stougaard and Molin [25]. Ethidium bromide was removed by repeated washings with CsCl₂-saturated 2-propanol and DNA was precipitated by 2 vol of ethanol at -40°C. Final DNA preparations were sterilized by filtration through Centrex™ cartridges (Schleicher and Schuell, Inc., Keene, NH)*.

Other Em^r plasmids tested for transforming ability were pAMβ1 [26] and pIP501 [27], with molecular masses of 26.5 and 30.7 kb, respectively. These large conjugative plasmids were isolated by the procedure mentioned above.

*Mention of a specific brand name does not imply endorsement by the authors or the U.S. Department of Agriculture to the exclusion of others not mentioned.

Preparation of cells for electroporation

Cultures tested for transformation with plasmid DNAs were incubated in Hogg-Jago-lactose (HJL) broth at 37°C for 16 h. Fresh transfers were made in HJL medium supplemented with 40 mM DL-threonine and cultures were incubated at 37°C until the culture reached an OD₆₆₀ of 0.2. 5 ml aliquots of cell suspensions were centrifuged at 10 000 × g for 10 min at 4°C and washed with 5 ml of 5 mM K₂HPO₄-KH₂PO₄ (pH 7.0). After centrifugation, cell pellets were dispersed in 1 ml of electroporation medium (EPM) which contained 5 mM K₂HPO₄-KH₂PO₄, 1 mM MgCl₂·6 H₂O and 0.3 M raffinose (pH 7.4). The cell suspension in EPM was kept at 4°C until use, usually within 60 min.

Electroporation conditions

To a 0.8 ml aliquot of cell suspension, transforming DNA (1–5 µg) in 50 µl of EPM was added. Electroporation was performed in a BioRad Gene Pulser™ apparatus (Bio-Rad Laboratories, Richmond, CA), according to the manufacturer's instructions. The Gene Pulser was set at 25 µF capacitance to deliver up to 2000 V (5 kV/cm) pulses. Immediately following the electroporation procedure, 0.1 vol of a 10 × HJL medium was added to the cell suspension which was then stored at 4°C for up to 24 h, before plating

in 10 ml of HJL agar. After solidifying, the bottom layer was overlaid with 10 ml of HJL agar containing 30 $\mu\text{g}/\text{ml}$ of erythromycin (Em). Plates were incubated at 37°C for 24–72 h before scoring EM^r transformants.

Electrophoretic analysis

Purified plasmid DNA isolated from Em^r transformants was analyzed by agarose gel electrophoresis under conditions described previously [24]. The molecular mass of plasmids was estimated with the aid of *Escherichia coli* V517 plasmids as standards [28].

Materials

All biochemicals were obtained from Sigma Chemical Co., St. Louis, MO. Restriction endonucleases were purchased from Bethesda Research Laboratories, Rockville, MD. Agarose was bought from FMC Corporation, Rockland, ME. All other chemicals and reagents were commercial products of the highest analytical purity. Microbiological medium components were purchased from Difco Laboratories, Detroit, MI.

Results

Effect of voltage and capacitance on cell viability and transformability

Voltage and capacitance settings selected for electroporation had a significant effect on cell viability. Results of detailed studies with *S. thermophilus* ST128 showed that cell viability (CFU = colony forming units/ml) was not affected until a combination of 25 μF capacitance and 1 kV (2.5 kV/cm) settings was used for single-hit electric pulsing (Fig. 1). At higher voltages, a progressive increase in the degree of cell death was observed, reaching 92% following exposure to a single pulse at 2 kV setting (5 kV/cm).

When early-log phase ($OD_{660} = 0.2$) cells of *S. thermophilus* ST128 were suspended in standard EPM and mixed with 5 μg of pVA736 as the transforming DNA, the number of transformants increased with voltage (Fig. 1), reaching a plateau at around 1.6 kV (4.0 kV/cm) instrument setting. At higher voltages, the number of Em^r transformants gradually declined.

Multiple-hit electric pulsing at 5 s intervals and 5 kV/cm voltage had a cumulative effect on culture viability of *S. thermophilus* ST108 (Table II). Nearly identical results were obtained with all other *S. thermophilus* strains listed in Table I.

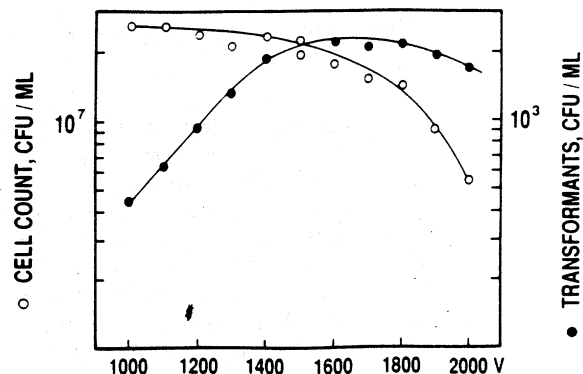


Fig. 1. Effect of voltage on cell viability and transformability of *S. thermophilus* ST128. Capacitance: 25 μF ; DNA: pVA736, 5 μg . Voltage values indicate Gene Pulser instrument settings.

Effect of pH

The effectiveness of electroporation in inducing the uptake of pVA736 by *S. thermophilus* was markedly influenced by the pH of the electroporation medium. Under standard electroporation conditions (i.e., Gene Pulser set at 1.6 kV and 25 μF) with the pH of EPM being the only variable, the highest number of transformants was obtained at pH 4.5 ($5 \times 10^3/\mu\text{g}$ of DNA). Between pH 5.0 and 8.5, the number of transformants averaged $3-5 \times 10^2/\mu\text{g}$ of DNA, whereas below pH 4.0, less than 100 transformants/ μg of DNA were recovered in Em-agar. All *S. thermophilus* strains included in this study responded to changes in medium pH in the same manner.

Table II. Effect of repeated pulsing on viability of *S. thermophilus* ST108.

No. of pulses	CFU/ml ^a	% survival
0	1.5×10^8	100
1	5×10^7	33
2	2.5×10^7	16
3	1.2×10^7	8
4	3.5×10^6	2.3
5	7×10^5	0.4

Electroporation conditions: voltage: 5 kV/cm; capacitance: 25 μF ; time constant: 3.6 ms; temperature: 4°C.

^aCFU = colony forming unit; after electroporation, cell suspensions were diluted serially and plated in HJL agar. Plates were scored after 48 h at 37°C.

Effect of buffer components on transformation

Changes in the composition of EPM influenced the number of transformants generated by the electroporation procedure. The replacement of K^+ with Na^+ in the buffer salts resulted in a 10-fold reduction of Em^r transformants for all *S. thermophilus* strains. The omission of $MgCl_2$ (1–5 mM) from EPM or its replacement with $CaCl_2$ also reduced the number of transformants to 10^2 or less per μg of DNA. On the other hand, substitution of raffinose with sucrose or reducing its concentration (0.1 M) failed to have a substantial effect on the number of Em^r transformants detected in Em -agar.

Responsiveness of *S. thermophilus* strains to electroporation

The effect of single-hit electric pulsing (4 kV/cm, 25 μF) on cell viability (CFU/ml) and transformation efficiency with pVA736 was found to be strain dependent (Table III). With the exception of strain ST134, all *S. thermophilus* strains were transformable with pVA736.

Table III. Effect of electroporation on the viability and transformability of *S. thermophilus* strains.

Strain	% loss of CFU/ml ^a	No. of Em^r transformants/ μg of DNA ^b
ST101	40	9×10^2
ST102	65	8.5×10^2
ST104	38	6×10^2
ST108	67	7.5×10^2
ST110	72	9×10^2
ST113	35	5.5×10^2
ST116	40	1.6×10^3
ST120	28	10^3
ST126	35	1.4×10^3
ST128	75	5×10^3
ST132	30	1.5×10^3
ST133	35	10^3
ST134	45	0
ST136	55	7×10^2

Electroporation conditions: voltage: 4 kV/cm; capacitance: 25 μF ; time constant: 3.6 ms.

Electroporation was done in EPM (pH 4.5).

^a and ^b Average values of 3 independent trials.

Em^r transformants resisted antibiotic concentrations higher than 100 μg /ml. The lack of transformability in strain ST134 may be due to high restriction endonuclease activity of undetermined specificity against pVA736 (our laboratory, unpublished observations).

Electroporation trials with 4 and 24 h old cultures of several strains of *S. thermophilus* demonstrated that culture age was not a significant factor in the number of attainable Em^r transformants. Electroporation of 24 h old *S. thermophilus* cultures routinely yielded 10^3 – 10^4 Em^r transformants/ μg of pVA736. Furthermore, supplementation of the growth medium (HJL) with DL-threonine which is known to have a mild destabilizing effect on cell wall biosynthesis in lactic acid bacteria [29], had a variable effect on transformability and increased the number of transformants by 15–20% in only 6 of the strains used in this study.

Electrophoretic analysis of plasmids isolated and purified from all *S. thermophilus* transformants showed the presence of a new, 7.6 kb entity (Fig. 2). This new plasmid in Em^r transformants showed a restriction endonuclease digestion pattern identical to that reported for the cloning vector pVA736 by Macrina *et al.* [4], with single restriction sites for *EcoRI*, *HindIII* and *KpnI*.

The results of plasmid analyses also confirmed the compatibility of pVA736 with several indigenous cryptic plasmids found in different strains of *S. thermophilus* [22], such as the plasmids pER8 (2.2 kb), pER13 (4.2 kb), pER36 (3.7 kb) and pER16 (4.5 kb).

Molecular mass of transforming DNA and electroporation

The electroporation technique was also suitable for introducing the larger Em^r conjugative plasmids pAM β 1 (26.5 kb) and pIP501 (30.7 kb) into *S. thermophilus*. However, with an increase in the size of the transforming plasmid, there was a concomitant decline in the transformability of ST128 (Table IV). Multiple-hit electric pulsing failed to influence the transformation frequency for any of the plasmid DNAs tested.

Effect of post-electroporation conditions

Different incubation and plating conditions were also tested in attempts to increase the number of transformants recovered in Em agar. Essentially no difference was found in the number of trans-

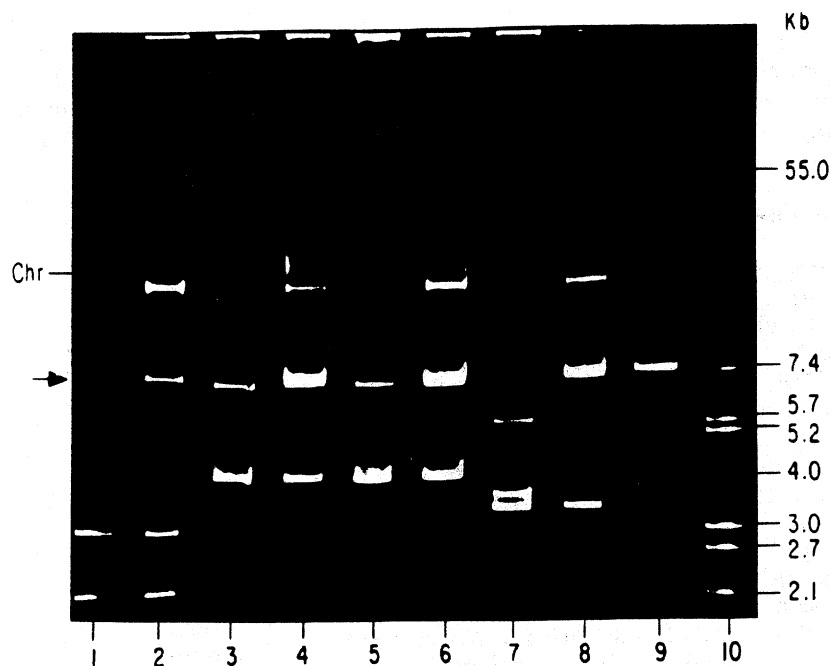


Fig. 2. Agarose gel electrophoresis profiles of plasmids isolated from *Em^r* transformants of *S. thermophilus*. 1: ST108/pER8 (2.2 kb); 2: ST108 *Em^r* transformant; 3: ST113/pER13 (4.23 kb); 4: ST113 *Em^r* transformant; 5: ST116/pER16 (4.46 kb); 6: ST116 *Em^r* transformant; 7: ST136/pER36 (3.7 kb); 8: ST136 *Em^r* transformant; 9: pVA736 (7.6 kb); 10: *E. coli* V517 plasmid standards. Arrow on the left indicates the relative position of pVA736 in the gel. Chr: chromosomal DNA. Additional bands visible in lanes 1–9 are partially uncoiled or open circular forms of plasmids listed above (see [22]).

formants between electrically pulsed transformation mixtures incubated at 4 or 25°C for up to 24 h before plating in single- or double-layer selective agar plates. However, in the case of the single-layer agar plating technique, a minimum incubation period of 3–4 h at 10 or 25°C was necessary before plating in *Em*–agar, in order to allow for the expression of the new plasmid pVA736 in *S. thermophilus* transformants. Alternatively, electrically-pulsed transformation mixtures were plated immediately in non-

selective agar and incubated for a minimum of 4 h or longer before overlaying with *Em*–agar. No significant difference in the total number of transformants was found between the two plating techniques.

Control experiments

The possibility of transforming *S. thermophilus* with pVA736 to erythromycin resistance without electroporation was also studied. The results of

Table IV. Effect of plasmid size on transformation frequency in *S. thermophilus* ST128^a.

pH	No. of transformants with ^b		
	pVA736 (7.6 kb)	pAMβ1 (26.5 kb)	IP501 (30.7 kb)
4.5	2500	110	15
7.5	750	30	3

^aElectroporation conditions: 4 kV/cm, 25 μF, single pulse; 5 μg of DNA.

^bAverage values calculated from 3 independent trials.

control trials indicated that without application of electric field pulses, no transformants were obtained with any of the *S. thermophilus* strains tested.

Discussion

To our knowledge, this is the first report on the transformability of *S. thermophilus* with plasmid DNA by electric field pulses. The electroporation technique is simple, rapid and reproducible, and DNA uptake is not dependent upon cell competence which is lacking in *S. thermophilus* and other lactic streptococci [30]. Furthermore, the method described here is applicable to many different strains of *S. thermophilus* and the uptake and expression of the molecular cloning vector pVA736 is apparently hindered only in strains with a high level of restriction endonuclease activity.

The efficiency of transformation by electroporation is influenced by several parameters. These include the combination of voltage and capacitance selected, the pH and composition of the electroporation medium, the molecular mass of the cloning vector employed, and post-electroporation incubation conditions. The age of the culture or its preconditioning with cell wall destabilizing agents, such as DL-threonine, appear to be less important factors.

A main advantage of the electroporation technique is that expression of introduced DNA can be measured within 24–48 h. In contrast, the polyethylene glycol-dependent transformation of protoplasts of lactic acid bacteria requires 5–7 days to allow for protoplast regeneration [31] which, in some cases, occurs in very low yields [7, 32]. Although protoplast regeneration in *S. thermophilus* is mentioned in earlier literature [33], successful genetic transformation of this species by a polyethylene glycol-assisted spheroplast transfection technique has been reported only recently [8].

The optimum voltage range for the transformation of *S. thermophilus* by electroporation is 3.75–4.25 kV/cm and with pVA736, the highest number of Em^r transformants attainable under conditions outlined here is 10^3 – 10^4 /μg of DNA. This value compares favorably with results published recently by Harlander [20] on the electroporation-dependent transformation of *S. lactis* with the plasmids pSA3 and pGB301.

In conclusion, our studies indicate that electroporation is a convenient and reproducible

technique for introducing DNA into intact cells of *S. thermophilus*, an organism known to be refractory to genetic transformation by classical methods. Predictably, electroporation will accelerate molecular cloning studies in this species and other lactic acid bacteria that are essential in food processing.

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